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EFFECTS OF DEGASIFIED EXTENDER ON QUALITY PARAMETERS OF CRYOPRESERVED BULL SPERMATOZOA

Megha Pande[1†] --- N. Srivastava[2] --- J.S. Rajoriya[3] --- S.K. Ghosh[4]--- J.K. Prasad[5]--- S.S. Ramteke[6]

[1,2]*ICAR-Central Institute for Research on Cattle, Meerut Cantt, Uttar Pradesh, India*

[3,4,5,6]*Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India*

ABSTRACT

One of the most important factors contributing to poor quality semen post thaw has been reported to be oxidative stress. Continued interaction of spermatozoa with dissolved oxygen in extender renders detrimental effect by generating reactive oxygen species during processing. This formed the basis to investigate the effects of removal of oxygen from the Tris-Glycerol-Egg Yolk (TG-EY) extender on the post-thaw semen quality. Semen ejaculates (n=26) collected from three cross bred bulls with mass activity of ≥3 and progressive motility of ≥70% were split in two fractions (control and treatment) to obtain 80 x 10^6 progressively motile sperm/mL of extended semen. The TG-EY extender composed of 3.028 g of Tris, 1.675 g of citric acid, 1.25 g of fructose, 7.0 mL of glycerol, 10 mL of egg yolk, 10^5 IU Penicillin G Sodium and 10^5 µg Streptomycin for 100 mL of deionized water for control and degasified (freeze-pump-thaw cycling method using liquid nitrogen) for treatment. Semen was processed and filled in 0.25 mL polyvinyl chloride straws, sealed and cryopreserved for at least 2 week before evaluation. Assessment of post-thaw motility, viability, acrosome and plasma membrane integrity of spermatozoa were evaluated after thawing at 37°C for 30s. Study revealed a significant (viability and acrosome integrity, $p<0.05$) and highly significant (post-thaw motility and plasma membrane integrity, $p<0.01$) improvement in all the semen quality parameters in degasified as compared to control group. This study showed that degasification of extender prior to their use can serve to improve semen quality to highly acceptable levels.

Keywords: Oxidative stress, Acrosome integrity, Cryopreservation, Plasma membrane integrity, ROS, Motility

† *Corresponding author*

Contribution/ Originality

This study shows importance of degasification of extender in cryopreservation protocol. The assays used to evaluate effect of degasification were currently in vogue. Fewer studies were available to evaluate the effect of degasification in cattle sperm cryopreservation. The paper contributes by showing the importance of degasification on sperm cryosurvival.

1. INTRODUCTION

Wider application of artificial insemination (AI) technology requires processing and cryopreservation of semen. For successful cryopreservation, the spermatozoa must retain its motility, plasma membrane configuration and integrity, enzymes like acrosin and hyaluronidase, DNA integrity and other characteristics. Disruption of any of these functions or abilities will significantly affect the fertilizing capacity of post-thaw semen. There are many factors responsible for success of a cryopreservation protocol including type of extender, interaction of components of extender with atmospheric oxygen and generation of reactive oxygen species (ROS), cooling rate, thawing rate, packaging as well as the individual animal variation [1, 2].

One of the most important factors contributing to poor quality semen post thaw has been reported to be oxidative stress. Freezing/thawing events of semen preservation are known to produce reactive oxygen species in semen samples. During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases the susceptibility to lipid peroxidation due to higher production of ROS [3]. Excessive production of ROS during freezing/thawing process has been associated with the reduced post-thaw motility, viability, membrane integrity, antioxidant status, and fertility and sperm functions [4].

There are several mechanisms proposed to account for the decline in sperm motility associated with oxidative stress peroxidation of polyunsaturated fatty acids (PUFA) in membrane lipid [5]. Sperm cells contain high concentrations of PUFA and therefore are highly susceptible to lipid peroxidation (LPO) leading to a subsequent loss of motility, membrane integrity, fertilizing capability and metabolic changes in sperm [6]. During cryopreservation PUFA in the sperm plasma membrane undergo peroxidation, which results in the formation of ROS catalyzed reaction [4]. Effect of oxidative stress is particularly important during cryopreservation.

The analysis of semen of mammalian species showed that the production of ROS and LPO occurrence is increased during freezing-thawing [7, 8]. The main site of ROS and LPO formation are mitochondria and sperm cell membranes [9], which are particularly vulnerable to damage from sudden temperature changes. Although, aerobic cells have substrates and enzymes to prevent or restrict the formation and propagation of ROS, but the antioxidant defense of spermatozoa are relatively weak and these germ cells are very susceptible to oxidative stress [10]. ROS are neutralized by the natural antioxidants in the semen [11] like superoxide dismutase (SOD) and glutathione peroxidase forming the enzyme defense mechanism protecting the sperm from peroxidation [12].

Sperm cells, when frozen and thawed for artificial insemination (AI) are exposed to oxygen and light radiation which could irreversibly affect sperm functions [13]. Long back in 1943, MacLeod documented the toxicity of oxygen on human spermatozoa. Spermatozoa loses motility more rapidly under 95% oxygen than under nitrogen but could be protected by catalase suggesting that sperm suspensions produce ROS [14].

Aerobic incubation periods led to an increase in lipid peroxidation and structural damage to the plasma membrane and are linked with decreased spermatozoa motility [10]. Moreover, availability of oxygen in the system is not an absolute governing force for spermatozoa metabolism [15]. Thus it was hypothesized that removal of oxygen (degasification) from the extender could help to improve quality of spermatozoa post thaw. This study gets justification from the fact that report on effects of reduced oxygen in extended semen on spermatozoa quality parameters like progressive motility, livability, acrosome and plasma membrane integrity and lipid peroxidation or antioxidant activities are not available.

2. MATERIALS AND METHODS

2.1. Animals

The present study was conducted at Germ-Plasm Centre (GPC), Animal Reproduction Division, Indian Veterinary Research Institute, Izatnagar, Bareilly. Three healthy cross bred (CB) breeding bulls maintained at GPC under uniform feeding and housing conditions were utilized for the study.

2.2. Diluent Preparation

The cryoprotective extender for the treatment used in this study was prepared a day before. It composed of 3.028 g of Tris, 1.675 g of citric acid, 1.25 g of fructose, 7.0 mL of glycerol, 10^5 IU Penicillin G Sodium and 10^5 µg Streptomycin for 100 mL of deionized water which was degasified by Freeze-Pump-Thaw cycling method. The semen diluents required for each batch of semen (25-30mL) was degassed by flash-frozen process using liquid nitrogen. In this process liquid nitrogen (*quantum sufficiat*) was poured in chilled diluents kept in 50 mL cryovial until it was completely frozen. A warm water bath (40ºC) was then used to thaw the frozen diluents from which bubbles of gas formed and escaped. Simultaneously handheld vacuum pump with a maximum vacuuming of 25 inch Hg (MV8000 Automotive Tune-up kit, Mityvac, Lincoln Helios, India) was applied, and the flask was sealed with paraffin wax. On the day of collection a small hole was made into the paraffin seal to add the egg yolk @ 10%. This degasified diluent was used for extension of semen of treatment group. The cryoprotective extender for the control group was not degasified, which was the only difference between two extenders for the treatments.

2.3. Semen Collection

Two consecutive semen ejaculates were collected from three cross bred bulls using artificial vagina as per the standard method. Collections were taken between 08.00 to 09.00 h, before feeding. Immediately after collection, the tubes containing semen were placed in the water bath maintained at 35 ºC and samples were evaluated for various, macro- (volume, color and consistency) and microscopic (mass activity, 0-5 scale, sperm concentration, percentage of motile spermatozoa, live-dead count and membrane integrity). Of the total of 30 samples, ejaculates (n=26) showing mass activity of +3 and above and spermatozoa with >70% progressive motility were used in the study.

2.4. Fresh Semen Evaluation

The fresh semen sample was evaluated for mass motility, sperm concentration, initial progressive motility, viability and membrane integrity of spermatozoa. The mass activity of the semen sample was determined by assessing the motility of the fresh ejaculated spermatozoa just after semen collection. This was assessed by placing a drop of fresh semen on a clean, grease free glass slide without cover slip mounted on a stage maintained at 37°C under low power of microscope and graded on the scale of 0 to +5. The semen samples showing mass activity of +3 and above were utilized for experimental work. The concentration of spermatozoa (in millions per mL) in the fresh semen was determined by semen quality analyzer (SQA-Vb; MES, Israel). The concentration was assessed with the aim of ascertaining the final dilution rate of semen. The motility was recorded as percentage of progressively motile spermatozoa after the extension of semen. This was assessed by placing a drop of semen diluted with Tris dilutor on a clean, grease free glass slide mounted on a stage maintained at 37°C and observed under 400 x magnifications after covering with a cover slip. The semen was extended to such an extent that approximately 15-20 spermatozoa were visible under the visual field of microscope. Samples with more than 70% motility only were selected for the study. The viability was estimated by differential staining techniques using Eosin-Nigrosin stain [16] in fresh and post thaw samples.

2.5. Semen Processing

After the evaluation of quality, the fresh semen was then divided into two equal fractions; one fraction was diluted with the extender for the control group and other with the extender for the treatment group to obtain 80 x 10^6 progressively motile sperm/mL. Semen was filled in 0.25 mL polyvinyl chloride straws (Bal Krishan Plasikrafts, India) and sealed with poly vinyl alcohol powder. The straws were kept in a bread box, three fourth of it filled with distilled water. The bread box was then kept in lower chamber of the refrigerator. The packed straws, kept in the bread box, were then subjected to a combined cooling cum equilibration period of 3 h by which the temperature of water in the bread box reached 5°C. Straws from each group were taken at this time for evaluation of progressive motility, viability and acrosome integrity of spermatozoa. The

remaining straws were then transferred immediately to cold handling cabinet maintained at 5°C where straws were dried on a filter paper and spread evenly over the semen freezing rack. The racks along with the straws were kept in automatic programmable freezer (Biological cell freezer, IMV technology, France) till temperature of straws reached - 145°C. Then straws were immersed directly into liquid nitrogen (-196°C) for storage. The straws were stored at least 2 week before evaluation.

2.6. Post-Thaw Semen Evaluation

Immediately after thawing at 37°C for 30 s, assessment of post-thaw motility of spermatozoa, viability, acrosome and plasma membrane integrity of spermatozoa were carried out.

2.7. Assessment of Acrosome Integrity

The acrosome integrity (per cent normal acrosome) was evaluated in Giemsa-stained smears [17]. The fixed smears were observed under microscope using oil immersion objective at 1000x magnification. A minimum of 200 spermatozoa were counted and the acrosomes were classified as acrosome intact or acrosome damaged.

2.8. Assessment of Biochemical Integrity of Plasma Membrane

The plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) described by Jeyendran, et al. [18]. Sperm swelling was assessed by placing 15 µL of well-mixed sample on a warm slide (37°C) under light microscopy at 400 x magnifications. The spermatozoa were classified in following four classes based on tail swelling pattern. Pattern A: no swelling, no membrane reaction; Pattern B: Swelling of the tip of the tail; Pattern C: different types of hair-pin like swelling pattern or swelling of the mid piece; Pattern D: Complete tail swelling. Spermatozoa displaying either pattern B, C or D were considered positive for HOST. At least 300 spermatozoa per slide (1 slide for each of 3 straws) were observed.

Table-1. Semen quality parameters of fresh semen sample of crossbred bulls (Mean ± SEM)

Semen quality parameters	n=26
Mass activity	3.96 ± 0.15
Concentration (10^6/ml)	855.45 ± 20.84
Progressive motility (%)	81.16 ± 1.36
Viability (%)	85.34 ± 1.26
Acr. integrity (%)	84.54 ± 0.97
HOST (%)	76.80 ± 1.29
Abnormalities (%)	8.42 ± 0.48

2.9. Statistical Analysis

The data pertaining to the semen quality parameters of fresh semen samples of crossbred bulls are presented as mean ± SEM. The differences in mean values between control and treatment groups are compared by t-test [19] using SPSS software version 17.0.

3. RESULTS AND DISCUSSION

In the present study from a total of 30 ejaculates, 26 ejaculates were evaluated to know the effect of degasification of extender on semen quality parameters post thaw.

3.1. Quality Parameters of Fresh Semen

The semen quality parameters of the fresh semen samples are presented bull wise in Table 1.

3.2. Quality Parameters of Frozen-Thawed Spermatozoa

The objective of present investigation was to investigate effect of degasification of extender on quality parameters of frozen-thawed spermatozoa. The results obtained are presented below.

The progressive motility, viability, acrosome integrity and bio-chemical integrity of spermatozoa were significantly ($p < 0.01$) higher at post-thaw stage in all the three bulls in degasified extender group (Table 2) indicating that in the present experimental conditions, degasified extender provided a better result in terms of motile sperms during post-thaw stage.

Table-2. Semen quality parameters of frozen-thaw semen sample of crossbred bulls (Mean ± SEM)

Group.	n	Motility	Livability (%)	Acr. integrity (%)	HOST (%)
Control	26	49.73 ± 1.65^{a}	57.62 ± 1.61^{a}	62.12 ± 1.85^{a}	50.30 ± 1.59^{a}
Treatment	26	60.26 ± 1.44^{b}	65.85 ± 1.21^{b}	67.92 ± 1.23^{b}	61.12 ± 1.41^{b}

Values with different superscripts in a column differ significantly ($p<0.01$)

Based on the findings of the present study, the degasified semen extender group gave better results in term of per cent motility, viability, membrane integrity and acrosome integrity in frozen-thawed crossbred bull spermatozoa than control group. The motility, viability, plasma membrane integrity and acrosome integrity are essential for predicting the fertilizing capacity of spermatozoa.

In the present study, O_2 gas was removed using Freeze-Pump-Thaw cycling process where the chilled semen diluent was degassed via flash-frozen process by directly pouring liquid nitrogen to the Tris extender. The N_2 gas addition is said to reduce the metabolic activity of the spermatozoa and displaces dissolved O_2 level in the medium [14]. Freezing during cryopreservation also halts the metabolic processes of the spermatozoa, allowing indefinite storage without a significant loss of fertility [20].

Earlier, it was shown that the storage of diluted semen under N_2 gas reduces the oxygen tension as well as substantially reduces the metabolic activity of spermatozoa without affecting the pH of the medium [21]. The Caprogen diluent typically contains catalase and is stored under N_2 gas to minimize the level of peroxide generated in the storage medium. Gassing the diluents with nitrogen reduces oxygen tension in the diluents, thereby inhibiting sperm motility without the need to lower pH [20].

Our experiment of using degassed diluent for semen storage has shown a marked improvement in post-thaw semen quality thereby proving that removal of oxygen from the system (extender, in this experiment) shall bestow positive beneficial effect on sperm cells. The rationale behind this may be that liquid N_2 flushing may have reduced the oxygen tension as well as substantially reduced the metabolic activity of spermatozoa. It may have also minimized the level of peroxide generation in the storage medium which needs further investigation.

With respect to the metabolic patterns of spermatozoa, the availability of oxygen in the system is not an absolute governing force. The presence of oxygen permits metabolic activity that could not possibly occur in its absence, but its presence does not demand that response [15] indicating that respiration is secondary and not an essential part of the metabolic function. In the absence of oxygen the rebuilding of ATP may occur by glycolysis and its presence both by respiration and glycolysis. However other factors control the degree to which oxidation provides the energy for motility. MacLeod's work [14] has also indicated that respiration is secondary and not an essential part of the metabolic function.

Damage due to oxidative stress is said to be surpassed by additions of antioxidants prior to freezing processes. It has been shown that under certain experimental conditions energy production from glycolysis sometimes continues as rapidly in the presence of oxygen as it does in its absence [15] so there should have been no harm in removing gasses from the diluents. Such measurements often require spermatozoa to be moved from their normal environment to one that can more easily be controlled. In spite of the great interest in this field and the publication of many scientific papers on the subject, little is known concerning the metabolic behavior of the spermatozoa in its absolute surrounding medium. It can be concluded from this investigation that degasification of extender exerts a positive effect on the post-thaw quality of spermatozoa.

3.2. Conflict of Interest Statement

The authors do not have any conflict of interests in this experiment.

4. ACKNOWLEDGEMENT

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